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THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 264, No. 17, Issue of June 15, pp. 10002-10007, 1989
Printed in U.S.A.

Antithrombin III-dependent Anti-prothrombinase Activity of Heparin and Heparin Fragments*

(Received for publication, November 8, 1988)

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Heparin and heparin fragments in the molecular mass range 1,700–20,000 Da were examined for their ability to accelerate the antithrombin III (AT III)-dependent inhibition of human factor Xa and the prothrombin converting complex (prothrombinase) *during* human prothrombin activation. The prothrombinase reaction was modeled by a 3-parameter 2-exponential equation to determine the initial rate of prothrombin activation and the pseudo-first order rate constants of inhibition of prothrombinase and *in situ* generated thrombin activity. The catalytic specific activities of the heparins increased with increasing molecular size for both the inhibition of prothrombinase and factor Xa. A 10-fold increase over the entire M_r range was found. In contrast to results obtained by others (Ellis, V., Scully, M. F., and Kakkar, V. V. (1986) *Biochem. J.* 233, 161–165; Barrowcliffe, T. W., Havercroft, S. J., Kemball-Cook, G., and Lindahl, U. (1987) *Biochem. J.* 243, 31–37), all the heparins showed a 5-fold higher rate of inhibition of factor Xa when compared with the inhibition of prothrombinase, indicating that the factor Va-mediated protection of factor Xa from inhibition by AT III/heparin is independent of the molecular size of the heparin. Our original approach has also revealed a hitherto unrecognized phenomenon, namely, in addition to the accelerating effect of the heparins on the rate of formation of the inactive AT III-factor Xa complex, heparins with M_r > 4,500 reduce the *initial* rate of thrombin generation in the presence of AT III in a concentration-dependent way. We hypothesize that the formation of the dissociable ternary AT III-heparin-factor Xa complex results in a (partial) loss of factor Xa activity towards its natural substrate prothrombin.

One of the essential zymogen activation reactions of the blood coagulation cascade leading to the formation of insoluble fibrin strands is the activation of prothrombin by prothrombinase, a Ca^{2+}-dependent complex composed of the serine protease factor Xa, the non-enzymatic cofactor factor Va, and negatively charged phospholipids (Ref. 1 and references therein).

A major inhibitor of coagulation is antithrombin III (AT III),[1] a plasma protein which forms equimolar complexes with serine proteases of the coagulation system. In the presence of heparin the rates of inactivation of the proteases are enhanced (2). Previous studies have monitored the rate constant of inactivation of free factor Xa as a function of the molecular weight of heparin (3, 4). It was shown that although the binding of factor Xa and AT III to the same heparin molecule is not a prerequisite for catalysis of the inactivation of this protease, as it is for the inactivation of thrombin, the rate of factor Xa inactivation does increase with increasing heparin molecular weight.

Studies in purified systems have demonstrated that the AT III-dependent rate of inactivation of factor Xa is reduced when factor Xa is bound to a phospholipid surface in the presence of factor V (5) or factor Va (6, 7) or when bound to activated platelets (8), *i.e.* factor Xa is protected from inactivation by AT III. It was also shown that factor Xa, as part of the prothrombinase complex, is protected from heparin-catalyzed inhibition by AT III (5, 7, 8). However, the effect of the molecular size of heparin on the rate constant of inactivation of prothrombinase, and consequently on the extent of protection, has not been extensively studied. Moreover, the data available are conflicting. In one study it was observed that when factor Xa is bound to activated platelets the protective effect diminishes with decreasing heparin molecular weight (8), whereas in a second study, where factor Xa was bound to a phospholipid surface in the presence of factor V, it appeared that the protective effect became more pronounced with decreasing molecular weight (5). The latter observation led to the postulation that the lack of correlation between *in vitro* anti-factor Xa activity and antithrombotic action of low molecular weight heparin is caused by different inhibitory actions on free factor Xa and on factor Xa in its more physiological form, *i.e.* part of the prothrombinase complex (5).

Because the heparin-catalyzed AT III-dependent inactivation of factor Xa, as part of the prothrombinase, is not thoroughly investigated, we further explored this inhibition of factor Xa. In an attempt to imitate physiological conditions of factor Xa inactivation as close as possible, we studied the effect of AT III and heparin on factor Xa during human prothrombin activation in the presence of excess factor Va and phospholipid. We developed a mathematical model which adequately describes the thrombin generation curves as a time-dependent function of the initial rate of prothrombin activation and the pseudo-first order rate constants of *in situ* inhibition of factor Xa and thrombin.

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[1] The abbreviations used are: AT III, antithrombin III; S2238, D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide dihydrochloride; S2337, benzoyl-L-isoleucyl-L-glutamyl-(γ-piperidyl)-L-glycyl-L-arginine-*p*-nitroanilide hydrochloride; HSA, human serum albumin; STI, soybean trypsin inhibitor; ISH, the Fourth International Standard for Heparin.

This approach made it possible to quantitatively assess the effect of heparin and heparin fragments on the inhibition of prothrombinase during prothrombin activation. We observed a correlation between molecular size of the heparin fractions and their catalytic specific activity/mol of AT III-binding site. Furthermore, we present evidence that, in addition to the progressive heparin-catalyzed reaction, heparins of higher molecular weight ($M_r > 6000$), but not of low molecular weight cause an immediate, AT III-dependent inhibition of prothrombinase activity.

EXPERIMENTAL PROCEDURES

Materials—The Fourth International Standard for Heparin (ISH) was obtained from the National Institute for Biological Standards and Control, London, United Kingdom. The preparation has an activity of 1780 international units of heparin/ampule of 9.2 mg (9).

The high molecular weight heparin (heparin fraction D, batch RB 20 292), the low molecular weight heparin (CY216, batch: P46 XH), and the synthetic pentasaccharide heparin (IC83.1423, batch: ILS 6 388 B) were kindly donated by Choay Institut, Paris, France. Heparin fraction D was isolated from a commercial pig mucosal heparin by ion-exchange chromatography on DEAE-Sephacel and has a mean molecular weight of 20,300. The isolation as well as the physicochemical and biological properties of heparin fraction D are described in detail elsewhere (10). The CY216 was prepared by ethanol extraction of a commercial pig mucosal heparin (11), the mean molecular weight is 4,500 (80% of the material has a molecular weight between 2,400 and 7,200; 50% of the material is composed of dodeca-, tetradeca-, and hexadecasaccharides) and the specific activities are 200 anti-factor Xa units/mg and 10–20 anti-thrombin units/mg dry weight (data provided by Choay Institut). The pentasaccharide was synthesized as previously described, has a molecular weight of 1,714, and an activity of 4,000 anti-factor Xa units/mg dry weight (12).

High affinity heparin oligosaccharides were prepared by AT III-affinity chromatography and gel chromatography of nitrous acid-depolymerized heparin (13), and were a gift of Dr. Lindahl, Swedish University of Agricultural Sciences, Uppsala, Sweden. The oligosaccharides used in this study were an octasaccharide fragment, a 10–14 and a 18–24 saccharide fragment. The (mean) molecular weights of these oligosaccharides were calculated with M_r = 300 for each saccharide unit (13).

The molar concentrations of all heparin preparations used in this study were determined by stoichiometric titration of AT III (1 μM) in 50 mM Tris-HCl (pH 7.5) and 100 mM NaCl, as monitored by the intrinsic protein fluorescence enhancement at λ_{em} = 345 and λ_{ex} 285 nm (14). The chromogenic substrates S2238 and S2337 were purchased from KabiVitrum AB, Stockholm, Sweden. The specific thrombin inhibitor hirudin was obtained from Sigma. All other protein preparations and the mixed phospholipid vesicles were prepared as previously described (15, 16).

Determination of Pseudo-first Order Rate Constants of Inhibition of Factor Xa—The inhibition of factor Xa by AT III and heparin was performed under pseudo-first order conditions. Factor Xa was added to a mixture of AT III, prothrombin, $CaCl_2$, phospholipid vesicles composed of 20 mol % phosphatidylserine and 80 mol % phosphatidylcholine, and heparin in 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, and 0.5 mg of HSA/ml at 37 °C. The final concentrations were 5.0 nM factor Xa, 0.40 μM AT III, 1.5 μM prothrombin, 3.0 mM $CaCl_2$, and 50 μM phospholipid. The final heparin concentrations varied between 1.0 and 20 nM. At timed intervals after the addition of factor Xa, 40-μl samples were transferred to cuvettes containing 0.46 ml of 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 20 mM EDTA, 0.22 mM S2337, 0.20 μM hirudin, and 0.5 mg of HSA/ml at 37 °C. Hirudin was present to prevent interference of thrombin formed in the first reagent mixture with the amidolytic factor Xa assay. After sampling into the substrate solution, the rate of AT III-dependent inactivation of factor Xa was sufficiently slow not to result in any measurable loss of enzymatic activity during the recording of the absorbance.

The rate of increase of absorbance at 405 nm was measured on a dual wavelength spectrophotometer (reference wavelength: 500 nm) and the residual factor Xa concentrations were calculated from a standard curve constructed under the same conditions. Residual factor Xa was exponentially plotted against reaction time and the pseudo-first order rate constants of inactivation of factor Xa were calculated as the slopes of these plots.

Prothrombin Activation in the Presence of AT III and Heparin—Human prothrombin was activated by simultaneously adding prothrombin and AT III to a mixture of factor Xa, factor Va, $CaCl_2$, phospholipid vesicles composed of 20 mol % phosphatidylserine and 80 mol % phosphatidylcholine, and heparin in 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, and 0.5 mg of HSA/ml at 37 °C. The final concentrations were 2.0 pM factor Xa, 0.60 nM factor Va, 3.0 mM $CaCl_2$, 50 μM phospholipid, 1.5 μM prothrombin, and 0.40 μM AT III. The final heparin concentrations varied between 1.3 and 50 nM. After 5 min of prothrombin activation, a small aliquot (3 μl) of soybean trypsin inhibitor (STI) was added to a final concentration of 1.0 mg/ml. After the addition of STI no further prothrombin activation occurred, which made it possible to determine the rate of inactivation of thrombin activity (15). At timed intervals during the experiment 20-μl samples were transferred to cuvettes containing 0.48 ml of 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 20 mM EDTA, 0.24 mM S2238, and 0.5 mg of HSA/ml at 37 °C. The amount of amidolytic thrombin activity formed was determined in the same way as described for factor Xa.

Kinetic Analysis of the Inhibition of Prothrombin Activation and Formed Thrombin Activity—We modeled the prothrombin activation in the presence of AT III and heparin by assuming that the observed generation of thrombin activity is the result of its virtual rate of formation (V_{+1}) and its rate of inactivation (V_{-1}).

$$V_{obs} = d[\text{thrombin}]/dt = V_{+1} - V_{-1} \quad (1)$$

Under pseudo-first order conditions the rate of inactivation of thrombin activity is described by:

$$V_{-1} = k_1\,[\text{thrombin}] \quad (2)$$

where k_1 is the observed pseudo-first order rate constant of inhibition of thrombin activity. Under our conditions the rate of prothrombin activation is directly proportional to the amount of prothrombinase present (*i.e.* factor Xa concentration):

$$V_{+1} = k_p[\text{Xa}] \quad (3)$$

where k_p is the turnover number, *i.e.* the number of moles of prothrombin converted per min/mol of factor Xa under optimum conditions. Under pseudo-first order conditions of inactivation of factor Xa, the time-dependent factor Xa concentration ($[\text{Xa}]_t$) can be modeled by:

$$[\text{Xa}]_t = [\text{Xa}]_0\exp(-k_2t) \quad (4)$$

where $[\text{Xa}]_0$ is the initial factor Xa concentration, k_2 is an apparent pseudo-first order rate constant of inactivation of factor Xa, and t is the reaction time. Assumption of simple pseudo-first order kinetics was sufficient to describe the inactivation of factor Xa during the bovine prothrombinase reaction by AT III or AT III and heparin (8). Substituting Equations 2, 3, and 4 into Equation 1 yields:

$$V_{obs} = d[\text{thrombin}]/dt = V_i\exp(-k_2t) - k_1[\text{thrombin}] \quad (5)$$

where V_i is the initial rate of prothrombin activation ($V_i = k_p\,[\text{Xa}]_0$). Integration of Equation 5 leads to the following

$$[\text{thrombin}]_t = V_i(\exp(-k_2t) - \exp(-k_1t))/(k_1 - k_2) \quad (6)$$

Equation 6 describes the observed thrombin activity ($[\text{thrombin}]_t$) in terms of initial rate of prothrombin activation (V_i), pseudo-first order rate constants of inhibition of thrombin activity (k_1), and factor Xa (k_2) and reaction time (t). A close look at the equation reveals that the rate constants of inhibition of thrombin and factor Xa are interchangeable. Thus we optimized our experimental setup by adding STI after 5 min of prothrombin activation. After the addition of STI at time t' ($V_{+1} = 0$) and integration of Equation 5:

$$[\text{thrombin}]_t = [\text{thrombin}]_{t'}\exp(-k_1(t - t')) \quad (7)$$

According to Equation 7 the rate constant of inhibition of thrombin activity can be unequivocally determined and hence also the rate constant of inhibition of factor Xa. Equations 6 and 7 were combined in a computer program, which made a non-linear least squares fit of the equations to our experimental thrombin activity data using a modified Gauss-Newton algorithm (17) and estimated the parameters V_i, k_1, and k_2 and the corresponding 95% confidence intervals, *i.e.* approximately 2 × S.E. of the estimates.

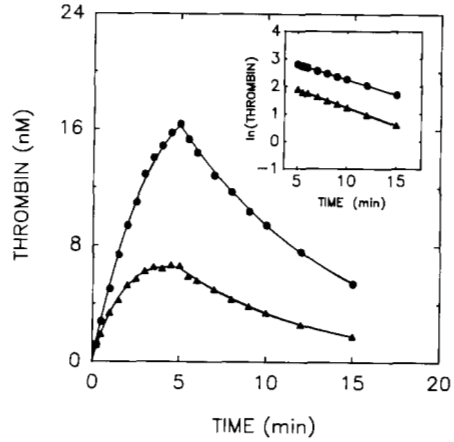

FIG. 1. **Time courses of thrombin activity.** Prothrombin was activated by factor Xa, factor Va, phospholipid vesicles composed of 20 mol % phosphatidylserine and 80 mol % phosphatidylcholine, and $CaCl_2$ as described under "Experimental Procedures," in the presence of AT III and 2.6 (●) or 10.5 nM (▲) ISH. STI was added at 5 min. The *inset* shows the semilogarithmic plots of the thrombin decay data obtained after the addition of STI. Equations 6 and 7 were fitted to the data and the resulting curves are indicated by the *solid lines.*

RESULTS

The Activation of Prothrombin in the Presence of AT III and Heparin—Prothrombin was activated in the presence of AT III and heparin as described under "Experimental Procedures." Fig. 1 shows typical examples of thrombin generation curves prior to and after the addition of STI. The amounts of ISH used were 2.6 and 10.5 nM. Equations 6 and 7 ("Experimental Procedures") were fitted to the experimental data, and the results are depicted as the *solid lines.* The inset in Fig. 1 shows a semilogarithmic replot of the decay data obtained after the addition of STI. This decay is monophasic, indicating that a single amidolytic active product is generated whose reaction with AT III should be taken into account, *viz.* meizothrombin (des fragment 1).[2] Clearly, the equations can be fitted to our experimental data very well. The residuals of the curves fitted to the experimental data varied randomly around 0 and were smaller than 2%.

The estimated parameters and their 95% confidence intervals were: $V_i = 6.2 \pm 0.2$ nM/min, $k_1 = 0.11 + 0.01$ min^{-1}, and $k_2 = 0.14 \pm 0.02$ min^{-1} in the presence of 2.6 nM ISH and $V_i = 4.0 \pm 0.2$ nM/min, $k_1 = 0.13 \pm 0.01$ min^{-1}, and $k_2 = 0.37 \pm 0.02$ min^{-1} in the presence of 10.5 nM ISH. The initial rate of prothrombin activation, determined in the absence of AT III and heparin was $V_i = 6.7$ nM/min. Hence, it appears that the initial rate of prothrombin activation is reduced in the presence of AT III and heparin. In addition, according to the pseudo-first order rate constants, the effect of AT III and heparin on the thrombin generation curves is found in the inactivation of factor Xa (k_2) rather than in the inactivation of thrombin (k_1). The effect of heparin and AT III on the initial rate of prothrombin activation, as well as on the pseudo-first order rate constants of inhibition of thrombin activity and factor Xa, will be further explored in the following sections.

The Effect of Heparin Fragments of Different Molecular Size on the Rate Constants of Inhibition of Prothrombinase during Prothrombin Activation—Pseudo-first order rate constants of factor Xa inhibition during prothrombin activation were obtained as a function of heparin concentration for the different heparin preparations, as outlined above. The rate constants were linearly dependent on the heparin concentrations studied. As an example, Fig. 2 shows the pseudo-first order rate constants of inhibition of prothrombinase and the matching 95% confidence intervals obtained in the presence of standard heparin.

The slopes of such plots, calculated by linear regression analysis, were determined and are shown in Fig. 3 as function of the heparin molecular weight, together with their 95% confidence intervals. The results indicate that up to M_r of approximately 6000 no clear relationship is seen between the acceleration of inhibition of prothrombinase and the molecular size of the heparin fragments. The observed differences between the rate constants approximate the 95% confidence intervals; hence the increasing tendency cannot be regarded as significant. However, at molecular weights higher than about 6000, a significant increase is noted, resulting in a 10-fold increase in catalytic efficiency over the entire molecular weight range (Table I).

Comparison between the Rate Constants of Inhibition of

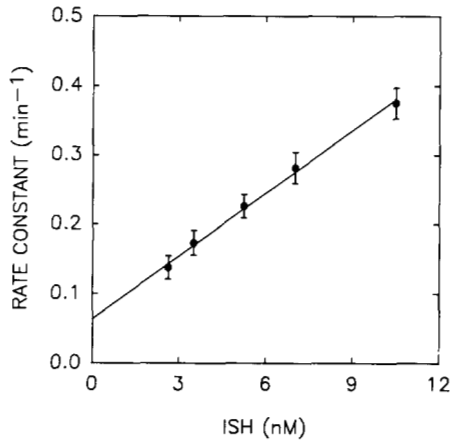

FIG. 2. **Rate constants of inactivation of factor Xa during prothrombin activation as a function of the concentration of standard heparin.** The pseudo-first order rate constants and the 95% confidence intervals were determined as described under "Experimental Procedures." The *solid line* is obtained by linear regression analysis of the data.

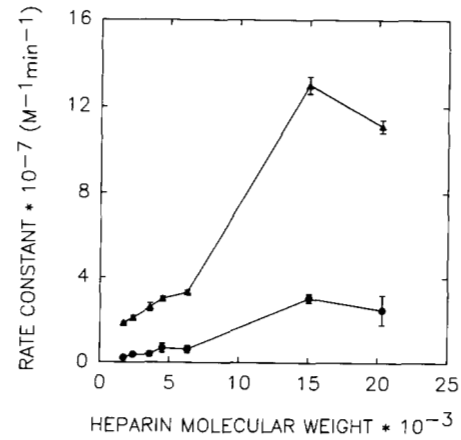

FIG. 3. **Rate constants of inactivation of prothrombinase during prothrombin activation (●) and of factor Xa (▲) as a function of the heparin molecular weight.** The rate constants per molar heparin concentration were determined as described in the text. Shown are the values of the rate constants with their 95% confidence intervals.

[2] Previous work from our laboratory (15) has shown that under the conditions of prothrombin activation used in this study, the major amidolytic active product is meizothrombin (des-fragment 1) which is, throughout this paper, referred to as thrombin activity.

Factor Xa and Prothrombinase—The heparin-dependent pseudo-first order rate constants of inhibition of factor Xa were determined as described under "Experimental Procedures." We reasoned that a comparison between the heparin-dependent inhibition of factor Xa and prothrombinase should be made under comparable conditions. Therefore the inhibition of factor Xa was determined in the presence of phospholipid, Ca^{2+}, and prothrombin. For each heparin fragment the rate constants were linearly dependent on the heparin concentration. The slopes of plots of the pseudo-first order rate constants as a function of the heparin concentrations were determined by linear regression analysis. The data and their 95% confidence intervals are also shown in Fig. 3 and listed in Table I. The data show that the rate constants of inhibition of factor Xa are related to the heparin molecular weight too. Below a molecular weight of about 6,300 a small, but significant increase of the acceleration of the inactivation of factor Xa is observed. Between a molecular weight of 6,300 and 15,000 the catalytic efficiency increases 4-fold.

When each of the nonenzymatic components were separately examined for an effect on the inhibition of factor Xa, we found, in keeping with earlier work on bovine factor Xa, that phospholipids do not alter the kinetics of inhibition (7). In contrast to the inhibition of bovine factor Xa by bovine AT III, Ca^{2+} ions stimulate the reaction of the human reactants in the presence of heparin (fractions). A slight stimulation (1.2-fold) was observed with heparin fragments of $M_r < 4500$. This stimulation increased with increasing molecular size of the heparin fragment; *i.e.* the catalytic efficiency of heparin fraction D increased 2.5-fold in the presence of 5 mM Ca^{2+}. However, the Ca^{2+}-enhanced accelerating ability was diminished when prothrombin was also present in the reaction mixture. We could demonstrate that prothrombin dose-dependently neutralizes the activity of heparins with $M_r > 6300$ (data not shown).

From the data listed in Table I it is seen that over the entire molecular weight range the catalytic specific activities of heparin fragments for the inhibition of factor Xa are about 5 times higher compared to their specific activities for the inhibition of prothrombinase. The fluctuation of the ratios of the specific activities is random, and lies within the experimental errors.

Heparin-stimulated Inhibition of Initial Rate of Prothrombin Activation—We initially assumed that it would be sufficient to apply a 2-parameter fit, revealing the rate constants of inhibition of thrombin (k_1) and factor Xa (k_2), to model our experimental thrombin generation data, and to regard the initial rate of prothrombin activation as a non-adjustable parameter. However, when V_i was determined in the absence of AT III and heparin and its value was used to compute k_1 and k_2, large deviations between the calculated thrombin generation data and the ones determined experimentally were observed in experiments where heparin molecules of a size greater than hexadecasaccharide were used. Hence, we did not use an independently determined value of V_i, but computed its value too. Fig. 4 shows the initial rates of prothrombin activation as revealed by the fit procedure, from thrombin generation curves obtained in the presence of AT III and heparin fraction D, the 4th International Standard for Heparin, or the heparin 18–24 saccharide fragment.

From these data it is seen that each of the three heparins causes a concentration-dependent decrease of the initial rate of prothrombin activation. Inhibition up to nearly 50% of the initial value can be obtained. The other heparin preparations, pentasaccharide, octasaccharide, 10–14 saccharide, and CY216, had no significant inhibitory effect on the initial rate of prothrombin activation in the range of heparin concentrations we studied.

We hypothesized that the observed anomaly between the rate, as determined in the absence of AT III and heparin, and the initial rate, revealed by the fitting of Equation 6 to our experimental thrombin generation data in the presence of AT III and heparins larger than hexadecasaccharide, may be accounted for by the instantaneous formation of the dissociable ternary complex between AT III, heparin, and factor Xa. The formation of this complex results in a (partial) loss of factor Xa activity towards its substrate prothrombin. To rule out the possibility that the observed decrease in the initial rate of prothrombin activation is an AT III-independent effect of heparin, we determined the rate of prothrombin activation in the absence of AT III, but in the presence of heparin. Thrombin generation was monitored and proceeded linearly for at least 5 min. We found that the rate of prothrombin activation was not affected by any of the heparin fractions used. Thus, the observed inhibition of the initial rate of prothrombin activation is dependent on the presence of both AT III and heparin.

In addition, the data shown in Fig. 4 suggest a linear decrease of the initial rate of prothrombin activation. From these plots we calculated a reduction of the initial rate of prothrombin activation of 0.38 nM thrombin/min/nM heparin fraction D, 0.26 nM thrombin/min/nM ISH, and 0.064 nM thrombin/min/nM 18–24 saccharide. Thus, the heparin-in-

TABLE I

Rate constants of the heparin-dependent inhibition of factor Xa and prothrombinase by AT III

Heparin type	M_r	Rate constant[a]	
		Factor Xa	Prothrombinase
		$\times 10^{-7} M^{-1}\ min^{-1}$	
Pentasaccharide	1,714	1.85 ± 0.05	0.23 ± 0.07
Octasaccharide	2,400	2.1 ± 0.1	0.37 ± 0.08
10–14 Saccharide	3,600	2.6 ± 0.2	0.39 ± 0.11
CY216	4,500	3.0 ± 0.1	0.68 ± 0.22
18–24 Saccharide	6,300	3.3 ± 0.1	0.62 ± 0.17
ISH	15,000	13.0 ± 0.4	3.03 ± 0.20
Heparin fraction D	20,300	11.1 ± 0.3	2.49 ± 0.70

[a] The observed pseudo-first order rate constant divided by the molar heparin concentration as determined by stoichiometric titration of AT III.

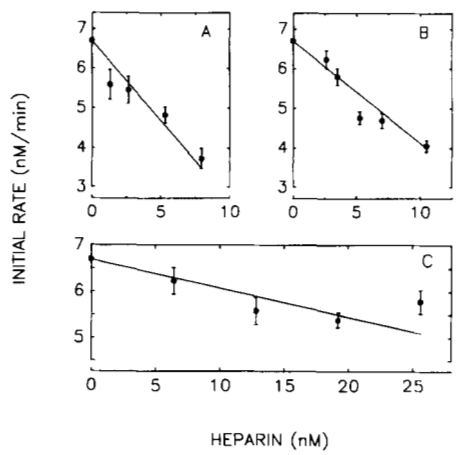

FIG. 4. **Initial rates of prothrombin activation as a function of heparin concentration.** The prothrombinase reaction was inhibited by 0.40 μM AT III in the presence of heparin fraction D (*A*), ISH (*B*), and 18–24 saccharide (*C*). The initial rates and the 95% confidence intervals were determined as described under "Experimental Procedures." In the absence of heparin and AT III the initial rate was 6.7 nM thrombin/min.

duced reduction of the initial rate of thrombin generation increases with increasing heparin chain length.

Effect of Heparin on the Inhibition of Prothrombinase-formed Thrombin Activity—The fitting of Equations 6 and 7 to the thrombin generation data also revealed the apparent pseudo-first order rate constants of inhibition of formed thrombin activity. We observed no dependence of the rate constants on the amount of heparin present for the different heparin preparations. All pseudo-first order rate constants varied randomly around a mean value of 0.137 min^{-1} with a maximum value of 0.197 min^{-1} and a minimum value of 0.103 min^{-1}. This observation is not unexpected, because we previously demonstrated that when prothrombin concentrations above 1 μM are used to measure prothrombinase activity, the major reaction product with amidolytic activity is meizothrombin (des-fragment 1), rather than α-thrombin, and at the time we showed that the AT III-dependent inhibition of meizothrombin (des-fragment 1) is not catalyzed by heparin (15).

DISCUSSION

The formation of the prothrombinase complex is essential for the generation of thrombin at a significant rate (1). AT III and heparin have a potentially important role in the regulation of prothrombinase by inhibiting its formation (inactivation of free factor Xa) and/or by inhibiting its activity once it is formed. Previous reports (5, 8) have indicated a differential activity of free factor Xa and prothrombinase in their heparin-catalyzed reaction with AT III. That is, factor Va and/or phospholipids appear to protect factor Xa from inhibition by AT III/heparin. However, conflicting results have been reported regarding the degree of protection (8, 18, 19) as well as on the effect of the molecular size of heparin fragments on the disparity between the rates of inhibition of free and complexed factor Xa. This disparity was found to increase (8) or decrease (5) with increasing molecular size of heparin.

Our experimental approach presented in this paper is different from the previously reported ones in that we studied the effect of heparin (fragments) and AT III on fully assembled prothrombinase during prothrombin activation in a well defined reaction system. We very adequately modeled the generation and inactivation of thrombin activity, which allowed the determination of the initial rate of prothrombin activation and the pseudo-first order rate constants of inhibition of generated thrombin activity and of prothrombinase.

Progressive Inhibition of Prothrombinase—The pseudo-first order rate constant of inhibition of prothrombinase during prothrombin activation in the presence of AT III varied linearly with the heparin concentration. Because the AT III concentration was much higher than the heparin concentrations and reported values for the dissociation constant of the heparin-AT III interaction (20, 21), it is quite possible that essentially all heparin fragments were saturated with AT III. The catalytic constant derived from the slopes of the lines of the rate constants plotted *versus* the molar concentration of heparin (mole AT III binding site/L) was about $3–6 \times 10^6$ M^{-1} min^{-1} for heparin fragments with M_r between 1700 and 6300. At molecular weights higher than 6300 the ability of heparin to enhance the inhibition of prothrombinase during prothrombin activation in the presence of AT III increases 4–6-fold. Our results indicate that, on a molar basis, heparin fragments with low M_r (<6000) are less effective than fragments of higher M_r in inhibiting prothrombinase.

Instantaneous Inhibition of Prothrombinase—Our approach to examining the inhibitory action of AT III/heparin during the prothrombinase reaction resulted in an, until now, unrecognized mode of action of AT III/heparin on prothrombinase-catalyzed thrombin generation. Evidence was obtained that in addition to the progressive inhibition of prothrombinase, AT III/heparin causes an immediate, partial neutralization of the prothrombinase activity. That is, the *initial* rate of prothrombin activation decreases with increasing amounts of heparin. Moreover, this direct inhibitory action was much more pronounced for heparin fragments with higher M_r than for low M_r heparins. In the absence of AT III none of the heparins studied inhibit prothrombin activation. This rules out that under these conditions heparin inhibits the prothrombinase reaction by disrupting the prothrombinase complex (22). We hypothesize that the initial, very rapid, formation of the ternary complex between factor Xa, heparin, and AT III directly removes factor Xa from the prothrombinase complex and thereby reduces the amount of prothrombinase available. Alternatively, the prothrombinase in the dissociable complex with heparin and AT III may have a reduced activity towards prothrombin. Unfortunately, an attempt to further reduce the initial rate of prothrombin activation by increasing the heparin concentration failed because the progressive inhibition of prothrombinase became too fast to give reliable thrombin generation data for the fit procedure.

Disparity between Rates of Inhibition of Factor Xa and Prothrombinase—The pseudo-first order rate constant of inhibition of factor Xa increases linearly with the concentration of the various heparin fragments. Thus we assumed that all heparin was saturated with AT III as was the case in the experiments with prothrombinase. The dependence of the catalytic constant on the molecular size of the heparin fragment agrees closely with the data reported for bovine factor Xa (4, 5). That is, the catalytic efficiencies of the heparin fragments, based on their AT III-binding molar concentration, decreased with decreasing molecular size. The scarce data available on human factor Xa (5) indicate that Ca^{2+} ions accelerate the factor Xa-AT III reaction 1.2-fold when unfractionated heparin was present but not in the case of oligosaccharide fragments. We confirmed that the Ca^{2+}-induced acceleration increased with increasing molecular size of the heparin fragments. However, this stimulation appears to be counterbalanced by the heparin neutralizing property of prothrombin.

Our data clearly indicate a higher catalytic activity of each heparin fragment for factor Xa inhibition than for the inhibition of prothrombinase. In contrast with the observation of Barrowcliffe *et al.* (5), heparin fragments of high M_r are unable to overcome the protective effect of factor Va on the inhibition of factor Xa. In fact, the disparity between the catalytic constants when compared under identical conditions was found to be independent of the molecular size of the heparin fragments.

In summary, the results presented in this paper suggest that a saccharide sequence, additional to the unique pentasaccharide sequence required for AT III binding (23), stimulates the inhibition of prothrombinase to the same degree as was observed for factor Xa in the absence of factor Va. In addition, such a non-AT III binding saccharide structure induces: 1) a second mode of action of heparin, *i.e.* a direct inhibition of the initial rate of thrombin generation and 2) a Ca^{2+}-stimulated inhibition of factor Xa.

At present it is unclear which properties of the additional saccharide sequence are responsible for our observations. Is it the length *per se* which gives these effects or is there an involvement of additional structural features of the heparin chain? In this respect, it has been demonstrated that the

bimolecular rate constant of the heparin-catalyzed inactivation of factor Xa by AT III is dependent on ionic strength in the presence of a high affinity M_r 8000 heparin, whereas an ionic strength independence is seen in the presence of a pentasaccharide (24). This observation is indicative of an electrostatic interaction between factor Xa and the M_r 8000 heparin. Thus, an effect of heparin charge density, which is well established for heparin-catalyzed thrombin inactivation (25, 26), may contribute to the heparin M_r effects we observed. However, we cannot rule out that after the first encounter of heparin and AT III in the pentasaccharide region, secondary interactions between heparin and AT III occur (27, 28), which may also contribute to our observations.

The catalytic constants were calculated on the basis of molar concentration of AT III-binding sites and all these sites were saturated with AT III. Therefore it is our conclusion that although simultaneous binding of AT III and factor Xa or prothrombinase to the same heparin molecule is not a prerequisite for the acceleration of inhibition, it does increase the catalytic efficiency of the heparin molecule. Moreover, linkage of protease-AT III-heparin interactions, as recently demonstrated for the reaction with thrombin (21) may also contribute to the involvement of heparin chain length in the heparin-catalyzed inhibition of prothrombinase by AT III.

Acknowledgment—We are grateful to Dr. U. Lindahl for providing the heparin oligosaccharide fragments.

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